



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07K 17/08</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/40791</b> <b>(43) International Publication Date:</b> 19 December 1996 (19.12.96)
<b>(21) International Application Number:</b> PCT/DK96/00249 <b>(22) International Filing Date:</b> 7 June 1996 (07.06.96) <b>(30) Priority Data:</b> 0639/95 7 June 1995 (07.06.95) DK <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> OLSEN, Arne, Agerlin [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsvaerd (DK). BECK, Thomas, Christian [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsvaerd (DK). <b>(74) Common Representative:</b> NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsvaerd (DK).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> MODIFICATION OF POLYPEPTIDES  <b>(57) Abstract</b>  The invention relates to a process for producing polyalkylene oxide-polypeptide conjugates with reduced allergenicity comprising the steps of: a) generating a polyalkylene oxide halogen formiate; and b) conjugating at least one polyalkylene oxide halogen formiate to attachment groups on the polypeptide. Further, the invention is also related to a polyalkylene oxide-polypeptide conjugate with reduced allergenicity, an activated polyalkylene oxide for conjugating polypeptides, and the use of said process for reducing the allergenicity of industrial polypeptides. Finally it is the object of the invention to provide the use of said polypeptide conjugate for a number of industrial purposes, such as the use in personal care products and in detergent compositions.		

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**Title:** Modification of polypeptides

## **FIELD OF THE INVENTION**

The present invention relates to a process for producing polyalkylene oxide-polypeptide conjugates with reduced allergenicity, a polyalkylene oxide-polypeptide conjugate with reduced allergenicity, an activated polyalkylene oxide for conjugating polypeptides, the use of said process for reducing the allergenicity of industrial polypeptides, the use of said polypeptide conjugate for a number of industrial purposes, such as the use in personal care products and in detergent compositions.

## **BACKGROUND OF THE INVENTION**

Due to the strongly extended use of polypeptides, such as especially proteins and enzymes, for industrial and pharmaceutical purposes an increasing number of people are daily exposed to polypeptides. This exposure may inflict problems for people having an enhanced inclination for eliciting an allergic response toward polypeptides or to people frequently in direct contact with relatively large amounts of polypeptides.

The latter group of people includes employees handling the manufacturing of products containing polypeptides, professionals, such as hair dressers, using products containing polypeptides, and to some extent also end-users of cosmetics and toiletry products.

For more than two decades scientists all over the world have made efforts to develop a technology making it possible to eliminate the immunogenicity, allergenicity, and/or antigenicity of polypeptides for therapeutic use. Even though the "perfect" technology has not yet been developed some good results have been achieved.

Most of the developed technologies involve chemical or genetic modification of polypeptides leading to larger and/or heavier polypeptide molecules. It seems that the molecular weight has an influence on the immune system's response towards polypeptides. However, today no precise commonly accepted explanation of the advantageous effect of larger and/or heavier polypeptide molecules exists. Further, it is possible to find exceptions from the rule. Even though a polypeptide such as e.g. plant pollen is both large and heavy it is known to cause an allergic response for certain people.

One of the technologies, which has shown good results on polypeptides for therapeutic uses, involves modification of polypeptides by means of covalent attachment of strands of polyalkylene oxides (PAO), such as polyalkylene glycol (PAG), to polypeptide molecules.

In general such chemical attachment of polyalkylene oxides (PAO) to polypeptides is recognized to lead to polypeptides having increased stability, increased resistance to proteolytic inactivation, reduced immunogenicity, antigenicity and/or allergenicity, and a low toxicity.

PAO-polymer polyethylene glycol (PEG) and methoxypolyethylene glycol (mPEG), capped on one end with a functional group and reactive with amines on the polypeptide molecule, are often used.

To effect covalent attachment of polyalkylene glycol (PAG) to a polypeptide the hydroxyl end-groups of the polymer must first be converted into reactive functional groups. This process is frequently referred to as "activation", and the product is called "activated", "derivatized" or "functionalized". To obtain a selective and specific attachment of the polymer strand to the polypeptide a linker molecule having a suitable coupling group is normally used.

Various PAG activation methods are known. One of the first commonly used methods involved activating the hydroxyl end-group of PAG using cyanuric chloride (Abuchowski et al., (1977), J. Biol. Chem., 252, 3578). However this method is not suitable for modifying polypeptide for foods and pharmaceuticals as cyanuric chloride (2,4,6-trichloro-s-triazine) is toxic and the activated PAG is non-specific, as it may react with polypeptides having functional groups other than amines, such as free essential cysteine or tyrosine residues.

Other methods commonly used for activation of insoluble polymers include activation of functional groups with cyanogen bromide, periodate, glutaraldehyde, diepoxides, epichlorohydrin, divenyldisulfone, carbodiimide, sulfonyl halides, trichlorotriazine etc. (see R.F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S.S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G.T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques". Academic Press, N.Y.). Some of the methods concern activation of insoluble polymers but are also applicable to activation of soluble polymers e.g. periodate, trichlorotriazine, sulfonylhalides, divenyldisulfone, carbodiimide etc. The functional groups being amino, hydroxyl, thiol, carboxyl, aldehyde or sulfhydryl on the polymer and the chosen attachment group on the protein must be considered in choosing the activation and conjugation chemistry.

Additional methods for activation of polymers can also be found in WO 94/17039, US patent no. 5,324,844, WO 94/18247, WO 94/04193, US patent no. 5,219,564, US patent no. 5,122,614, WO 90/13540 (Enzon), and US patent no. 5,281,698 (Cetus), and more WO 93/15189 (Veronese) and for conjugation between activated polymers and enzymes e.g. Coagulation Factor VIII (WO 94/15625), haemoglobin (WO 94/09027), oxygen carrying molecule (US patent no. 4,412,989), ribonuclease and superoxide dismu-

tase (Veronese et al., (1985), App. Biochem. Biotech., 11, p. 141-45).

Summarized the polymer activation research has been concentrated on finding activated PAO-polymers: 1) being reactive under mild process conditions, 2) having a high degree of selectivity towards specific attachment groups on the polypeptide, and 3) which, during the conjugation process release only non-toxic residues.

Poly(ethylene glycol) succinoyl-N-hydroxysuccinimide ester (SS-PEG) (Abuchowski et al., (1984), Cancer Biochem. Biophys., 7, 175-186) is an example of such an activated PAO releasing only non-toxic residues, which are readily separable from the PAO-polypeptide conjugate. However the product has limited stability in aqueous media.

The above described polypeptide conjugation technology is designed to modify polypeptides for therapeutic use, for which selectiveness and controlled specific attachment are important. First of all because pharmaceutical polypeptides are often to be introduced into the bloodstream of the human body, which obviously lines up very high demands to the quality of the product. Secondly because pharmaceutical products have to be approved by national health authorities, such as e.g. the Food and Drug Administration (FDA) in the United States of America, who demand highly controlled production processes and thorough testing of the products.

#### Prior art patents

US patent no. 4,179,337 discloses a process for conjugation of polyalkylene glycol (PEG) to polypeptides for therapeutic use, such as insulin and certain enzymes, all having a physiological activity. The preparations of conjugated polypeptides have reduced immunogenicity and have further a prolonged lifetime in the bloodstream as compared to the parent polypeptides. The surplus amounts of polymer necessary to conjugate the polypep-



tide makes the method expensive.

WO 90/13540 (Enzon Inc.) concerns activated PEG derivatives, namely, poly(ethylene glycol) succinimide carbonates (SC-PEG) and the bifunctional derivative of PEG, namely, poly(ethylene glycol-bis-succinimide carbonate (BSC-PEG). Furthermore, the heterobifunctional derivatives of PEG, which in one end have the succinimide carbonate group and in the other end have a group which readily reacts with amino groups of proteins to afford PEG attachment through stable urethane linkages.

Furthermore, WO 90/13540 discloses a process for synthesis of SC-PEG via PEG-chloroformate by treating PEG with phosgene ( $O=C-Cl_2$ ). The resulting PEG-chloroformate is then reacted with N-hydroxysuccinimide (HOSu) followed by triethylamine (TEA) to yield the desired activated SC-PEG. During protein modification SC-PEG only releases non-toxic material (N-hydroxysuccinimide).

EP 632 082 (Heyleclina) describes the preparation of activated (methoxy)poly(alkylene glycol) carbamate (C-(m)PEG) from (m)PEG via (m)PEG-chloroformate. The described activated (m)PEG carbamate has a high degree of selectivity, as it only reacts with amine groups. In the application it is asserted that (m)PEG chloroformate readily reacts with amines, alcohols, phenols, and carboxylic acids.

#### Discussion of prior art

According to prior art it is known to conjugate polypeptides to a number of polyalkylene oxides (PAO), including polyalkylene glycols (PAG), such as polyethylene glycols (PEG). The general method involves activating a PAO-polymer to obtain a functionalized group which selectively attaches to a specific chemical group on the polypeptide. The selective attachment is advantageous in connection with pharmaceuticals, as pharmaceuticals often are to be introduced into the human bloodstream. However, the demand for selectivity and specific attachment makes the

polypeptide modification process cumbersome, slow, and reduces the process yield. Even though the selectiveness of the activated PAO does not affect the advantageous properties of the reaction end product in a negative manner, it certainly will affect the cost of the process, as it is necessary to add a surplus amount of activated PAO-polymer to obtain a suitable absolute process yield.

It is to be understood that conjugation of polypeptides which are not intended to enter the circulatory system of the human body, is a totally different matter, as such polypeptides are not intended to elicit a physiological response. Further, unintended exposure of the human body to such polypeptides will not involve direct contact with the bloodstream, as the polypeptide is not e.g. injected into the human circulatory system or intramuscular. The contact is much less pronounced, and when occurring, it will take place in the form of inhalation of polypeptides through the mouth, or as nasal or dermal contact.

The urge to obtain selective attachment of the polymer to the polypeptide is less important in the context of polypeptides for non-therapeutic purposes, as the demand for selectivity is a consequence of the "in-the-body" use.

Further, polypeptides for pharmaceutical purposes is produced in amounts of a few kilograms, while industrial polypeptides is produced in amounts of many 1000 kilograms. Techniques used for producing polypeptides for therapeutic purposes cannot always advantageously be adapted for producing polypeptides for industrial purposes.

Therefore, it would be desirable to be able to obtain PAO-polypeptide conjugates for purposes where the polypeptide is not intended to enter the body, the conjugate of which has reduced allergenicity using a minimum of process steps.



**SUMMARY OF THE INVENTION**

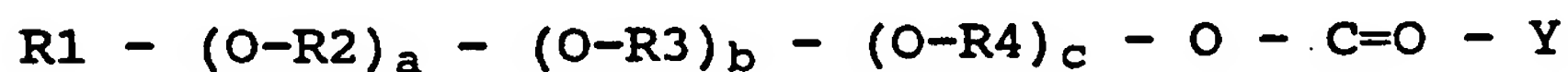
It is the object of the present invention to provide polyalkylene oxide-polypeptide conjugates for industrial purposes having reduced allergenicity.

The present inventors have surprisingly found an advantageous process for producing industrial polypeptides having reduced allergenicity using less process steps than expected.

In the first aspect the invention relates to a process for producing polyalkylene oxide-polypeptide conjugates with reduced allergenicity, using a polyalkylene oxide (PAO) as the starting material, comprising the steps of

- a) generating a polyalkylene oxide halogen formiate, and
- b) conjugating at least one polyalkylene oxide halogen formiate to attachment groups on the polypeptide.

Further the invention is directed towards an activated polyalkylene oxide capable of linking to attachment groups on a polypeptide, whose activated PAO has the generic structure



wherein

R1 is hydrogen, methyl, hydroxyl or methoxy,

R2 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

R3 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

R4 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

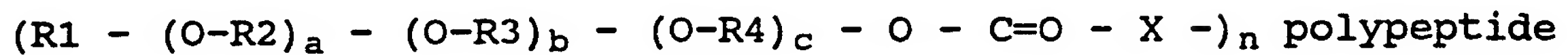
a is an integer between 1 and 1000,

b is an integer between 0 and 1000,

c is an integer between 0 and 1000, and

Y is a halogen or a nitrile.

Also contemplated according to the invention is polyalkylene oxide-polypeptide conjugates having the general formula



wherein

R1 is hydrogen, methyl, hydroxyl or methoxy,

R2 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

R3 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

R4 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

a is an integer between 1 and 1000,

b is an integer between 0 and 1000,

c is an integer between 0 and 1000,

n is an integer between 1 and 100, characterized in that X is a coupling group between the polymer and an polypeptide, which has been formed by reaction with a polyalkylene oxide halogen formiate.

Finally the invention relates to the use of the above process for reducing the allergenicity of polypeptides for industrial purposes, which include the use in personal care products and detergents.

#### BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows the results of the IT study in Brown Norway rats intratracheally exposed to immunized

1: Carezyme© core (CC)

2: Core Carezyme©-PEG 5,000 (CC/chlPEG5000)

3: Glycine-PEG 15,000 (Gly/SPEG15000)

4: 0.9% NaCl (NaCl)

The sera IgG and IgE antibodies were raised against Carezyme©

core

Figure 2 as figure 1 except that the sera IgG and IgE antibodies were raised against Carezyme© core-mPEG 5,000

Figure 3 shows the results of the IT study in Brown Norway rats intratracheally exposed to immunized:

- 1: Subtilisin Novo without PMSF (SN)
- 2: Subtilisin Novo with PMSF (SN-PMSF)
- 3: Subtilisin Novo-mPEG 15,000 (SN/SPEG15000)
- 4: Glycine-PEG 15,000 (Gly/SPEG15000)
- 6: 0.9% NaCl (NaCl)

The serum IgG and IgE antibodies were raised against Subtilisin Novo without PMSF.

Figure 4 shows as figure 3 except that the sera IgG and IgE antibodies were raised against Subtilisin Novo with PMSF.

Figure 5 as figure 3 except that the sera IgG and IgE antibodies were raised against Subtilisin Novo-mPEG 15,000.

#### DETAILED DESCRIPTION OF THE INVENTION

It is the object of the invention to provide a process for conjugating polyalkylene oxides and polypeptides to obtain an industrial product with reduced allergenicity.

In the context of the present invention "industrial polypeptides" are defined as polypeptides, such as proteins and enzymes, which are not to be introduced into the circulatory system of the body of humans and animals. Examples of such polypeptides, which are specifically contemplated, are polypeptides used in products such as detergents, household article products, agrochemicals, personal care products, such as cosmetics, toiletries, oral and dermal pharmaceuticals, composition use for processing textiles, compositions for

cleaning hard surfaces, compositions used for manufacturing food and feed etc.

The "circulatory system" of the body of humans and animals means, in the context of the present invention, the system which mainly consists of the heart and blood vessels. The heart delivers the necessary energy for maintaining blood circulation in the vascular system. The circulation system functions as the organisms transportation system, when the blood transports  $O_2$ , nutritious matter, hormones, and other substances of importance for the cell regulation into the tissue. Further the blood removes  $CO_2$  from the tissue to the lungs and residual substances to e.g. the kidneys. Furthermore the blood is of importance for the temperature regulation and the defence mechanisms of the body, which include the immune system.

As described above it is known to produce polyalkylene oxide-polypeptide conjugates by first generating a polyalkylene glycol chloroformate by using phosgene ( $Cl_2-C=O$ ) and thereafter reacting it with N-hydroxysuccinimide (HOSu) followed by triethylamine (TEA) to yield the desired activated PEG-derivative.

The above conjugation process is advantageous for producing polypeptides for therapeutic purposes, as a selective attachment to amine groups on the polypeptides will be obtained. However the process is cumbersome and slow, and costly.

In the first aspect the present invention relates to a process for producing polyalkylene oxide-polypeptide conjugates using a polyalkylene oxide (PAO) as the starting material. The conjugation process comprises the steps of:

- a) generating a polyalkylene oxide halogen formiate, and
- b) conjugating at least one polyalkylene oxide halogen formiate

to attachment groups on the polypeptide.

In comparison to the corresponding prior art process mentioned above, at least one process step has been eliminated, and still a product with reduced allergenicity is obtained.

#### Polymer

According to the invention the polyalkylene oxide (PAO) can be an polyalkylene glycol (PAG), such as an methoxypolyalkylene glycol (mPAG).

More specifically the PAG may advantageously be a polyethylene glycol (PEG), such as a methoxypolyethylene glycol (mPEG).

PAO-polymers having a molecular weight ( $M_r$ ) between 1 kDa and 60 kDa, such as between 2 kDa and 35 kDa, especially between 2 kDa and 25 kDa, may be used according to the invention.

Note that all polymer molecular weights mentioned in this application are average molecular weights.

#### Advantages of the invention

Polypeptides for industrial purposes need not attach to specific attachment groups on the polypeptide. Actually it may in certain cases even be an advantage to obtain a less specific attachment, as the attachment of PAO will be more homogeneous, and the addition of a large surplus amount of PAO is not needed. Further, the process runs much faster, and is less cumbersome to control.

Summarized, the process cost per kilogram is reduced in comparison with the corresponding prior art process for producing therapeutic polypeptides, which of course is especially important when processing very large amounts of polypeptides.

According to the above described prior art document EP 0 632

082-A1 (see page 19, line 41) mPEG chloroformate readily reacts with a number of chemical groups.

This is a simplification, as this is only correct when the reaction takes place in organic solvent. In an aqueous reaction solution chloroformate will mainly react (and attach) with amine groups on the polypeptide and reaction with (attachment to) other groups will be much less pronounced. Therefore, when using the process of the invention a conjugation product consisting of PAO-polymers conjugated to a number of chemical groups on the polypeptide can be obtained, and if desired a conjugation product mainly consisting of PAO-polymers conjugated to the amine groups on the polypeptide can be obtained.

Consequently the process of the invention gives the possibility of obtaining a specifically designed conjugated PAO-polypeptide product. The conjugation product is dependant on the reaction medium.

The present inventors have developed the process of the invention especially for large scale processing of industrial polypeptides.

Another advantage of the invention is that the prepared polypeptide-polymer conjugate has an improved stability in comparison to the parent polypeptide.

The process has all of the above mentioned advantages, and only a minimum of process steps are used to obtain polypeptides with reduced allergenicity, in comparison to the corresponding prior art processes.

#### Immunogenicity, antigenicity and allergenicity

"Immunogenicity" is a wider term than "antigenicity" and "allergenicity", and expresses the immune systems response to the presence of foreign substances. Said foreign substances are



called immunogens, antigens and allergens depending of the type of immune response the elicit.

An "immunogen" may be defined as a substance which, when introduced into circulatory system of animals and humans, is capable of stimulating an immunologic response.

The term "antigen" refers to substances which by themselves are capable of generating antibodies when recognized as a non-self molecule.

Further, an "allergen" may be defined as an antigen which may give rise to allergic sensitization or an allergic response by IgE antibodies (in humans, and molecules with comparable effects in animals).

It is, in the context of enzymes for industrial applications, important to distinguish between allergens mediating allergic responses e.g. intradermally, and respiratory allergens causing allergic responses by contact with cell-bound IgE in the respiratory tract.

Assessment of the allergenicity may be made by inhalation tests, comparing the effect of intratracheally (into the trachea) administered parent polypeptides with the corresponding polypeptides with reduced allergenicity modified according to the invention.

A number of *in vitro* animal models exist for assessment of the allergenicity of polypeptides. Some of these models give a suitable basis for hazard assessment in man. Suitable models include a guinea pig model and a rat model. These models seek to identify respiratory allergens as a function of elicitation reactions induced in previously sensitized animals. According to these models the alleged allergens are introduced intratracheally into the animals.

A suitable strain of guinea pigs, the Dunkin Hartley strain, does not as humans, produce IgE antibodies in connection with the allergic response. However, they produce another type of antibody the IgG1A and IgG1B (see e.g. Prentø, ATLA, 19, p. 8-14, 1991), which are responsible for their allergenic response to inhaled polypeptides including proteases. Therefore when using the Dunkin Hartley animal model, the relative amount of IgG1A and IgG1B is a measure of the allergenicity level.

A rat strain suitable for intratracheal exposure to polypeptides and enzymes is the Brown Norway strain. Brown Norway rats produce IgE as the allergic response.

Other animals such as rabbits may also be used for comparable studies.

In Example 6 it is shown that by using the process of the invention the allergenicity of polypeptides, such as the enzymes Carezyme® core and Subtilisin Novo, is reduced.

#### Polypeptides

A polypeptide having "reduced allergenicity" according to the invention indicates that the amount of produced IgE (in humans, and molecules with comparable effects in specific animals), which can lead to an allergic state, is significantly decreased when inhaling a modified polypeptide of the invention in comparison to the corresponding parent polypeptide.

The polypeptide to be conjugated according to the invention may be of plant, animal or microbial origin, although the polypeptides preferably is of microbial origin, such as of bacterial or fungal origin.

In an embodiment of the invention the polypeptide is a protein having a biological activity, such as anti-microbial activity.

In a preferred embodiment of the invention the protein is an

enzyme selected from the group of proteases, lipases, transferases, carbohydrases, oxidoreductases, and phytases.

Especially contemplated is enzymes with a molecular weight in the range between about 10 kDa and 100 kDa.

In the cases of the polypeptide having a biological or enzymatic activity, said activity is substantially maintained.

A "substantially" maintained activity is in the context of the present invention defined as an activity which is at least between 20% and 30%, preferably between 30% and 40%, more preferably between 40% and 60%, better from 60% up to 80%, even better from 80% up to about 100%, in comparison to the activity of the parent polypeptide.

The maximal number of polymer molecules which can be conjugated to the polypeptides depend to the number of attachment groups on the polypeptide. In general between 1 and 25 polymer molecules, preferably from 1 to 10 polymer molecules, are conjugated to each polypeptide molecule. This is significantly less than corresponding prior art techniques. Consequently the expense to polymers is reduced. To some extent it entails that the activity of the enzyme is substantially retained, as it is to be anticipated that the activity to a certain extent varies inversely with the number and the size of polymers conjugated to the polypeptide.

The polyalkylene oxide halogen formiate generated in step a) is preferably a polyalkylene oxide chloroformiate, such as methoxypolyalkylene glycol chloroformiate, and may be generated by using phosgene.

In Example 1 and Example 2 the preparation of a 15,000 and 5,000 methoxypolyethylene glycol chloroformiate, respectively, is described. However, according to the invention the halogen may also be any other halogen, such as Br or F.

In step b) of the process of the invention the attachment groups on the polypeptide can be any group selected from the group including amines, hydroxyls, alcohols, phenols, or carboxylic acids.

More specifically the polyalkylene oxide halogen formate may if desired attach to the side chain of e.g. serine, threonine, tyrosine, lysine, arginine, aspartate, glutamate in the polypeptide chain.

Another aspect of the invention is to provide an activated polyalkylene oxide (PAO) capable of linking to attachment groups on a polypeptide, which activated PAO has the generic structure



wherein

R1 is hydrogen, methyl, hydroxyl or methoxy,

R2 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

R3 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

R4 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

a is an integer between 1 and 1000,

b is an integer between 0 and 1000,

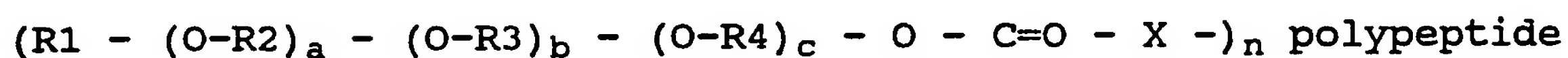
c is an integer between 0 and 1000, and

Y is a halogen or nitrile

The halogen Y may be either of Cl, Br, or F, although Cl is preferred.

The activated PAO of the invention may preferably have a molecular weight in the range of from about 1 kDa to 60 kDa, preferably from about 2 kDa to 35 kDa, especially from about 2 kDa to 25 kDa.

It is also the object of the invention to provide a polyalkylene oxide-polypeptide conjugate having the general formula



wherein R1, R2, R3, R4, a, b and c are defined as above. n is an integer between 1 and 100 and X is a coupling group between the polymer and an polypeptide.

The coupling group has been formed by reacting the activated PAO (polyalkylene oxide halogen formiate) of the invention with an attachment group on the polypeptide. Said attachment group may be e.g. an amine, hydroxyl, alcohol, phenol, and/or carboxylic acid on the polypeptide.

The attachment group may constitute the side chain of serine, threonine, tyrosine, lysine, arginine, aspartate, glutamate in the polypeptide chain.

Preferably the polypeptide conjugate of the invention is produced by the process of the invention.

In a preferred embodiment of the invention R2, R3 and R4 are CH<sub>2</sub>-CH<sub>2</sub>, CH<sub>2</sub>-CH-CH<sub>3</sub>, or CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>.

Activated PAO-polymers having a molecular weight (M<sub>r</sub>) between 1 and 60 kDa may be used as starting material for the process of the invention. Preferred are polymers having a molecular weight (M<sub>r</sub>) of between 2 kDa and 35 kDa, especially between 2 kDa and 25 kDa.

The polypeptide to be conjugated is preferably a protein having a biological activity or an enzyme as mentioned above.

A PAO-polypeptide conjugate of the invention has a total mo-

molecular weight in the range of 50 kDa to 250 kDa, preferably between 80 and 200 kDa.

A conjugate of the invention can be stored by freezing e.g. at about 18°C.

#### Compositions

The invention also relates to compositions comprising at least one polypeptide conjugate of the invention.

The composition may further comprise other enzymes/polypeptides and/or ingredients normally used in e.g. detergents, including soap bars, household articles, agrochemicals, personal care products, such as cleaning preparations e.g. for contact lenses, cosmetics, toiletries, oral and dermal pharmaceuticals, compositions used for treating textiles, compositions for cleaning hard surfaces, compositions used for manufacturing food, e.g. baking, and feed etc.

Examples of said enzymes/polypeptides include proteases, lipases, oxidoreductases, carbohydrases, transferases, such as transglutaminases, anti-microbial polypeptides, and phytases.

#### **Detergent Compositions**

According to the invention, the enzymes/polypeptides may typically be a component of a detergent composition, e.g., a laundry detergent composition or a dishwashing detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethylene glycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from



12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or non-aqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, non-ionic, cationic, or amphoteric (zwitterionic). The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzene-sulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of non-ionic surfactant such as alcohol ethoxylate (AEO or AE), alcohol propoxylate, carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more enzymes/polypeptides, such as amylases, pullulanase, esterase, lipase, cutinase, protease, cellulase, peroxidase, or oxidase, e.g., laccase, and anti-microbial polypeptides. One, more or all these enzymes/polypeptides may be modified according to the invention.

Normally the detergent contains 1-65% of a detergent builder, but some dishwashing detergents may contain even up to 90% of a detergent builder, or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

The detergent builders may be subdivided into phosphorus-containing and non-phosphorous-containing types. Examples of phosphorus-containing inorganic alkaline detergent builders include the water-soluble salts, especially alkali metal pyrophosphates, orthophosphates, polyphosphates and phosphonates. Examples of non-phosphorus-containing inorganic builders include water-soluble alkali metal carbonates, borates and silicates as well as layered disilicates and the various types of water-insoluble crystalline or amorphous alumino silicates of which zeolites is the best known representative.

Examples of suitable organic builders include alkali metal, ammonium or substituted ammonium salts of succinates, malonates, fatty acid malonates, fatty acid sulphonates, carboxymethoxy succinates, polyacetates, carboxylates, polycarboxylates, aminopolycarboxylates and polyacetyl carboxylates.

The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, polymaleates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent composition may contain bleaching agents of the

chlorine/bromine-type or the oxygen-type. The bleaching agents may be coated or encapsulated. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite or hypobromite as well as chlorinated trisodium phosphate. The bleaching system may also comprise a  $H_2O_2$  source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylene-diamine (TAED) or nonanoyloxybenzenesulfonate (NOBS).

Examples of organic chlorine/bromine-type bleaches are heterocyclic N-bromo and N-chloro imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric and dichloroisocyanuric acids, and salts thereof with water solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable. The bleaching system may also comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

In dishwashing detergents the oxygen bleaches are preferred, for example in the form of an inorganic persalt, preferably with a bleach precursor or as a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, persilicates and perphosphates. Preferred activator materials are TAED or NOBS.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708. The enzymes of the invention may also be stabilized by adding reversible enzyme inhibitors, e.g., of the protein type as described in EP 0 544 777 B1.

The detergent may also contain other conventional detergent

ingredients such as, e.g., fabric conditioners including clays, deflocculant material, foam boosters/foam depressors (in dishwashing detergents foam depressors), suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, dehydrating agents, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of laundry detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	7	-	12%
Alcohol ethoxysulfate (e.g. C <sub>12-18</sub> alcohol, 1-2 EO) or alkyl sulfate (e.g. C <sub>16-18</sub> )	1	-	4%
Alcohol ethoxylate (e.g. C <sub>14-15</sub> alcohol, 7 EO)	5	-	9%
Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	14	-	20%
Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	2	-	6%
Zeolite (as NaAlSiO <sub>4</sub> )	15	-	22%
Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	-	6%
Sodium citrate/citric acid (as C <sub>6</sub> H <sub>5</sub> Na <sub>3</sub> O <sub>7</sub> /C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> )	0	-	15%
Sodium perborate (as NaBO <sub>3</sub> ·H <sub>2</sub> O)	11	-	18%
TAED	2	-	6%
Carboxymethylcellulose	0	-	2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0	-	3%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001 - 0.5%		
Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0	-	5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
Alcohol ethoxysulfate (e.g. C <sub>12-18</sub> alcohol, 1-2 EO or alkyl sulfate (e.g. C <sub>16-18</sub> ))	1 - 3%
Alcohol ethoxylate (e.g. C <sub>14-15</sub> alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	15 - 21%
Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1 - 4%
Zeolite (as NaAlSiO <sub>4</sub> )	24 - 34%
Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	4 - 10%
Sodium citrate/citric acid (as C <sub>6</sub> H <sub>5</sub> Na <sub>3</sub> O <sub>7</sub> /C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> )	0 - 15%
Carboxymethylcellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001 - 0.5%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%



3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	5	-	9%
Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO)	7	-	14%
Soap as fatty acid (e.g. C <sub>16-22</sub> fatty acid)	1	-	3%
Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	10	-	17%
Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	3	-	9%
Zeolite (as NaAlSiO <sub>4</sub> )	23	-	33%
Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	-	4%
Sodium perborate (as NaBO <sub>3</sub> ·H <sub>2</sub> O)	8	-	16%
TAED	2	-	8%
Phosphonate (e.g. EDTMPA)	0	-	1%
Carboxymethylcellulose	0	-	2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0	-	3%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001	-	0.5%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0	-	5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzenesulfonate (calculated as acid)	8	- 12%
Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO)	10	- 25%
Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	14	- 22%
Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1	- 5%
Zeolite (as NaAlSiO <sub>4</sub> )	25	- 35%
Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	- 10%
Carboxymethylcellulose	0	- 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1	- 3%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001	- 0.5%
Minor ingredients (e.g. suds suppressors, perfume)	0	- 5%

5) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO or C <sub>12-15</sub> alcohol, 5 EO)	12	- 18%
Soap as fatty acid (e.g. oleic acid)	3	- 13%
Alkenylsuccinic acid (C <sub>12-14</sub> )	0	- 18%
Aminoethanol	8	- 18%
Citric acid	2	- 8%
Phosphonate	0	- 3%
Polymers (e.g. PVP, PEG)	0	- 3%
Borate (as B <sub>4</sub> O <sub>7</sub> )	0	- 2%
Ethanol	0	- 3%
Propylene glycol	8	- 14%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001	- 0.5%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0	- 5%

6) An aqueous structured liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	3	- 9%
Soap as fatty acid (e.g. oleic acid)	3	- 10%
Zeolite (as NaAlSiO <sub>4</sub> )	14	- 22%
Potassium citrate	9	- 18%
Borate (as B <sub>4</sub> O <sub>7</sub> )	0	- 2%
Carboxymethylcellulose	0	- 2%
Polymers (e.g. PEG, PVP)	0	- 3%
Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0	- 3%
Glycerol	0	- 5%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001	- 0.5%
Minor ingredients (e.g. dispersants, suds suppressors, per- fume, optical brighteners)	0	- 5%

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Fatty alcohol sulfate	5	- 10%
Ethoxylated fatty acid monoethanol- amide	3	- 9%
Soap as fatty acid	0	- 3%
Sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	5	- 10%
Soluble silicate (as $\text{Na}_2\text{O}, 2\text{SiO}_2$ )	1	- 4%
Zeolite (as $\text{NaAlSiO}_4$ )	20	- 40%
Sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	2	- 8%
Sodium perborate (as $\text{NaBO}_3 \cdot \text{H}_2\text{O}$ )	12	- 18%
TAED	2	- 7%
Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1	- 5%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001	- 0.5%
Minor ingredients (e.g. optical brightener, suds suppressors, per- fume)	0	- 5%

8) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	8	- 14%
Ethoxylated fatty acid monoethanol- amide	5	- 11%
Soap as fatty acid	0	- 3%
Sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	4	- 10%
Soluble silicate (as $\text{Na}_2\text{O}, 2\text{SiO}_2$ )	1	- 4%
Zeolite (as $\text{NaAlSiO}_4$ )	30	- 50%
Sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	3	- 11%
Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ )	5	- 12%
Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1	- 5%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001	- 0.5%
Minor ingredients (e.g. suds suppressors, perfume)	0	- 5%



9) A detergent composition formulated as a granulate comprising

Linear alkylbenzenesulfonate (calculated as acid)	6	- 12%
Nonionic surfactant	1	- 4%
Soap as fatty acid	2	- 6%
Sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	14	- 22%
Zeolite (as $\text{NaAlSiO}_4$ )	18	- 32%
Sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	5	- 20%
Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ )	3	- 8%
Sodium perborate (as $\text{NaBO}_3 \cdot \text{H}_2\text{O}$ )	4	- 9%
Bleach activator (e.g. NOBS or TAED)	1	- 5%
Carboxymethylcellulose	0	- 2%
Polymers (e.g. polycarboxylate or PEG)	1	- 5%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001	- 0.5%
Minor ingredients (e.g. optical brightener, perfume)	0	- 5%

10) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15	- 23%
Alcohol ethoxysulfate (e.g. C <sub>12-15</sub> alcohol, 2-3 EO)	8	- 15%
Alcohol ethoxylate (e.g. C <sub>12-15</sub> al- cohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	3	- 9%
Soap as fatty acid (e.g. lauric acid)	0	- 3%
Aminoethanol	1	- 5%
Sodium citrate	5	- 10%
Hydrotrope (e.g. sodium toluensulfonate)	2	- 6%
Borate (as B <sub>4</sub> O <sub>7</sub> )	0	- 2%
Carboxymethylcellulose	0	- 1%
Ethanol	1	- 3%
Propylene glycol	2	- 5%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001	- 0.5%
Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0	- 5%

## 11) An aqueous liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	20	- 32%
Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	6	- 12%
Aminoethanol	2	- 6%
Citric acid	8	- 14%
Borate (as B <sub>4</sub> O <sub>7</sub> )	1	- 3%
Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer)	0	- 3%
Glycerol	3	- 8%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001	- 0.5%
Minor ingredients (e.g. hydrotropes, dispersants, perfume, optical brighteners)	0	- 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25	- 40%
Nonionic surfactant (e.g. alcohol ethoxylate)	1	- 10%
Sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	8	- 25%
Soluble silicates (as $\text{Na}_2\text{O}$ , $2\text{SiO}_2$ )	5	- 15%
Sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	0	- 5%
Zeolite (as $\text{NaAlSiO}_4$ )	15	- 28%
Sodium perborate (as $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$ )	0	- 20%
Bleach activator (TAED or NOBS)	0	- 5%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001	- 0.5%
Minor ingredients (e.g. perfume, optical brighteners)	0	- 3%

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by (C<sub>12</sub>-C<sub>18</sub>) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C <sub>12</sub> -C <sub>18</sub> ) alkyl sulfate	9	- 15%
Alcohol ethoxylate	3	- 6%
Polyhydroxy alkyl fatty acid amide	1	- 5%
Zeolite (as NaAlSiO <sub>4</sub> )	10	- 20%
Layered disilicate (e.g. SK56 from Hoechst)	10	- 20%
Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	3	- 12%
Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	0	- 6%
Sodium citrate	4	- 8%
Sodium percarbonate	13	- 22%
TAED	3	- 8%
Polymers (e.g. polycarboxylates and PVP)	0	- 5%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001	- 0.5%
Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0	- 5%

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C <sub>12</sub> -C <sub>18</sub> ) alkyl sulfate	4	- 8%
Alcohol ethoxylate	11	- 15%
Soap	1	- 4%
Zeolite MAP or zeolite A	35	- 45%
Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	2	- 8%
Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	0	- 4%
Sodium percarbonate	13	- 22%
TAED	1	- 8%
Carboxymethyl cellulose	0	- 3%
Polymers (e.g. polycarboxylates and PVP)	0	- 3%
Enzymes including modified enzymes (calculated as pure enzyme protein)	0.0001	- 0.5%
Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0	- 3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature, 369, (1994), p. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary alcohol, a builder system (e.g.



phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

Particular forms of dishwashing detergent compositions within the scope of the invention include:

1) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	0.4	- 2.5%
Sodium metasilicate	0	- 20%
Sodium disilicate	3	- 20%
Sodium triphosphate	20	- 40%
Sodium carbonate	0	- 20%
Sodium perborate	2	- 9%
Tetraacetythylenediamine (TAED)	1	- 4%
Sodium sulphate	5	- 33%
Enzymes including modified enzymes	0.0001	- 0.5%

## 2) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant (e.g. alcohol ethoxylate)	1	- 2%
Sodium disilicate	2	- 30%
Sodium carbonate	10	- 50%
Sodium phosphonate	0	- 5%
Trisodium citrate dihydrate	9	- 30%
Nitrilotrisodium acetate (NTA)	0	- 20%
Sodium perborate monohydrate	5	- 10%
Tetraacetylenediamine (TAED)	1	- 2%
Polyacrylate polymer (e.g. maleic acid/acrylic acid co-polymer)	6	- 25%
Enzymes including modified enzymes	0.0001	- 0.5%
Perfume	0.1	- 0.5%
Water	5	- 10

## 3) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	0.5	- 2.0%
Sodium disilicate	25	- 40%
Sodium citrate	30	- 55%
Sodium carbonate	0	- 29%
Sodium bicarbonate	0	- 20%
Sodium perborate monohydrate	0	- 15%
Tetraacetylenediamine (TAED)	0	- 6%
Maleic acid/acrylic acid copolymer	0	- 5%
Clay	1	- 3%
Poly(amino acids)	0	- 0%
Sodium polyacrylate	0	- 8%
Enzymes including modified enzymes	0.0001	- 0.5%

## 4) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	1	- 2%
Zeolite MAP	15	- 42%
Sodium disilicate	30	- 34%
Sodium citrate	0	- 12%
Sodium carbonate	0	- 20%
Sodium perborate monohydrate	7	- 15%
Tetraacetylenediamine (TAED)	0	- 3%
Polymer	0	- 4%
Maleic acid/acrylic acid copolymer	0	- 5%
Organic phosphonate	0	- 4%
Clay	1	- 2%
Enzymes including modified enzymes	0.0001	- 0.5%
Sodium sulphate	Balance	

## 5) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	1	- 7%
Sodium disilicate	18	- 30%
Trisodium citrate	10	- 24%
Sodium carbonate	12	- 20%
Monopersulphate (2 $\text{KHSO}_5 \cdot \text{KHSO}_4 \cdot \text{K}_2\text{SO}_4$ )	15	- 21%
Bleach stabilizer	0.1	- 2%
Maleic acid/acrylic acid copolymer	0	- 6%
Diethylenetriaminepentaacetate, pentasodium salt	0	- 2.5%
Enzymes including modified enzymes	0.0001	- 0.5%
Sodium sulphate, water	Balance	

## 6) POWDER AND LIQUID DISHWASHING COMPOSITION WITH CLEANING SURFACTANT SYSTEM

Nonionic surfactant	0	- 1.5%
Octadecyl dimethylamine N-oxide dihydrate	0	- 5%
80:20 wt.C18/C16 blend of octadecyl dimethylamine N-oxide dihydrate and hexadecyldimethyl amine N-oxide dihydrate	0	- 4%
70:30 wt.C18/C16 blend of octadecyl bis (hydroxyethyl)amine N-oxide anhydrous and hexadecyl bis (hydroxyethyl)amine N-oxide anhydrous	0	- 5%
C <sub>13</sub> -C <sub>15</sub> alkyl ethoxysulfate with an average degree of ethoxylation of 3	0	- 10%
C <sub>12</sub> -C <sub>15</sub> alkyl ethoxysulfate with an average degree of ethoxylation of 3	0	- 5%
C <sub>13</sub> -C <sub>15</sub> ethoxylated alcohol with an average degree of ethoxylation of 12	0	- 5%
A blend of C <sub>12</sub> -C <sub>15</sub> ethoxylated alcohols with an average degree of ethoxylation of 9	0	- 6.5%
A blend of C <sub>13</sub> -C <sub>15</sub> ethoxylated alcohols with an average degree of ethoxylation of 30	0	- 4%
Sodium disilicate	0	- 33%
Sodium tripolyphosphate	0	- 46%
Sodium citrate	0	- 28%
Citric acid	0	- 29%
Sodium carbonate	0	- 20%
Sodium perborate monohydrate	0	- 11.5%
Tetraacetylenethylenediamine (TAED)	0	- 4%
Maleic acid/acrylic acid copolymer	0	- 7.5%
Sodium sulphate	0	- 12.5%
Enzymes including modified enzymes	0.0001	- 0.5%

## 7) NON-AQUEOUS LIQUID AUTOMATIC DISHWASHING COMPOSITION

Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0	- 10.0%
Alkali metal silicate	3.0	- 15.0%
Alkali metal phosphate	20.0	- 40.0%
Liquid carrier selected from higher glycols, polyglycols, polyoxides, glycolethers	25.0	- 45.0%
Stabilizer (e.g. a partial ester of phosphoric acid and a C <sub>16</sub> -C <sub>18</sub> alkanol)	0.5	- 7.0%
Foam suppressor (e.g. silicone)	0	- 1.5%
Enzymes including modified enzymes	0.0001	- 0.5%

## 8) NON-AQUEOUS LIQUID DISHWASHING COMPOSITION

Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0	- 10.0%
Sodium silicate	3.0	- 15.0%
Alkali metal carbonate	7.0	- 20.0%
Sodium citrate	0.0	- 1.5%
Stabilizing system (e.g. mixtures of finely divided silicone and low molecular weight dialkyl polyglycol ethers)	0.5	- 7.0%
Low molecule weight polyacrylate polymer	5.0	- 15.0%
Clay gel thickener (e.g. bentonite)	0.0	- 10.0%
Hydroxypropyl cellulose polymer	0.0	- 0.6%
Enzymes including modified enzymes	0.0001	- 0.5%
Liquid carrier selected from higher glycols, polyglycols, polyoxides and glycol ethers	Balance	



## 9) THIXOTROPIC LIQUID AUTOMATIC DISHWASHING COMPOSITION

C <sub>12</sub> -C <sub>14</sub> fatty acid	0	- 0.5%
Block co-polymer surfactant	1.5	- 15.0%
Sodium citrate	0	- 12%
Sodium tripolyphosphate	0	- 15%
Sodium carbonate	0	- 8%
Aluminium tristearate	0	- 0.1%
Sodium cumene sulphonate	0	- 1.7%
Polyacrylate thickener	1.32	- 2.5%
Sodium polyacrylate	2.4	- 6.0%
Boric acid	0	- 4.0%
Sodium formate	0	- 0.45%
Calcium formate	0	- 0.2%
Sodium n-decydiphenyl oxide disulphonate	0	- 4.0%
Monoethanol amine (MEA)	0	- 1.86%
Sodium hydroxide (50%)	1.9	- 9.3%
1,2-Propanediol	0	- 9.4%
Enzymes including modified enzymes	0.0001	- 0.5%
Suds suppressor, dye, perfumes, water	Balance	



## 10) LIQUID AUTOMATIC DISHWASHING COMPOSITION

Alcohol ethoxylate	0	- 20%
Fatty acid ester sulphonate	0	- 30%
Sodium dodecyl sulphate	0	- 20%
Alkyl polyglycoside	0	- 21%
Oleic acid	0	- 10%
Sodium disilicate monohydrate	18	- 33%
Sodium citrate dihydrate	18	- 33%
Sodium stearate	0	- 2.5%
Sodium perborate monohydrate	0	- 13%
Tetraacetythylenediamine (TAED)	0	- 8%
Maleic acid/acrylic acid copolymer	4	- 8%
Enzymes including modified enzymes	0.0001	- 0.5%

## 11) LIQUID AUTOMATIC DISHWASHING COMPOSITION CONTAINING PROTECTED BLEACH PARTICLES

Sodium silicate	5	- 10%
Tetrapotassium pyrophosphate	15	- 25%
Sodium triphosphate	0	- 2%
Potassium carbonate	4	- 8%
Protected bleach particles, e.g. chlorine	5	- 10%
Polymeric thickener	0.7	- 1.5%
Potassium hydroxide	0	- 2%
Enzymes including modified enzymes	0.0001	- 0.5%
Water	Balance	

11) Automatic dishwashing compositions as described in 1), 2), 3), 4), 6) and 10), wherein perborate is replaced by percarbonate.

12) Automatic dishwashing compositions as described in 1) - 6) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in

"Efficient manganese catalysts for low-temperature bleaching", Nature, 369, (1994), p. 637-639.

### **Personal care applications**

Also for personal care products the conjugated enzymes with reduced allergenicity of the invention are of interest.

#### Proteases

Proteases are well-known active ingredients for cleaning of contact lenses. They hydrolyze the proteinaceous soil on the lens and thereby makes it soluble. Removal of the protein soil is essential for the wearing comfort.

Proteases are also effective ingredients in skin cleaning products, where they remove the upper layer of dead keratinaceous skin cells and thereby make the skin look brighter and more fresh.

Proteases are also used in oral care products, especially for cleaning of dentures, but also in dentifrices.

Further, proteases are used in toiletries, bath and shower products, including shampoos, conditioners, lotions, creams, soap bars, toilet soaps, and liquid soaps.

#### Lipases

Lipases can be applied for cosmetic use as active ingredients in skin cleaning products and anti-acne products for removal of excessive skin lipids, and in bath and shower products such as creams and lotions as active ingredients for skin care.

Lipases can also be used in hair cleaning products (e.g. shampoos) for effective removal of sebum and other fatty material from the surface of hair.

Lipases are also effective ingredients in products for cleaning of contact lenses, where they remove lipid deposits from the

lens surface.

### Oxidoreductases

The most common oxidoreductase for personal care purposes is an oxidase (usually glucose oxidase) with substrate (e.g. glucose) that ensures production of  $H_2O_2$ , which then will initiate the oxidation of for instance  $SCN^-$  or  $I^-$  into antimicrobial reagents ( $SCNO^-$  or  $I_2$ ) by a peroxidase (usually lactoperoxidase). This enzymatic complex is known in nature from e.g. milk and saliva.

It is utilized commercially as anti-microbial systems in oral care products (mouth rinse, dentifrice, chewing gum), where it also can be combined with an amyloglucosidase to produce the glucose. These systems are also known in cosmetic products for preservation.

Anti-microbial systems comprising the combination of an oxidase and a peroxidase are known in the cleaning of contact lenses.

Another application of oxidoreductases is oxidative hair dyeing using oxidases, peroxidases and laccases .

Free radicals formed on the surface of the skin (and hair) known to be associated with the ageing process of the skin (spoilage of the hair).

The free radicals activate chain reactions that lead to destruction of fatty membranes, collagen, and cells.

The application of free radical scavengers such as Superoxide dismutase into cosmetics is well-known (R. L. Goldemberg, DCI, Nov. 93, p. 48-52).

Protein disulfide isomerase (PDI) is also an oxidoreductase. It can be utilized for waving of hair (reduction and reoxidation of disulfide bonds in hair) and repair of spoiled hair (where the damage is mainly reduction of existing disulfide bonds).

### Carbohydrases

Plaque formed on the surface of teeth is composed mainly of polysaccharides. They stick to the surface of the teeth and the microorganisms. The polysaccharides are mainly  $\alpha$ -1,6 bound glucose (dextran) and  $\alpha$ -1,3 bound glucose (mutan). The application of different types of glucanases such as mutanase and dextranase helps hydrolysing the sticky matrix of plaque, making it easier to remove by mechanical action.

Also other kinds of biofilm for instance the biofilm formed in lens cases can be removed by the action of glucanases.

### Anti-microbial polypeptides

Anti-microbial polypeptides have widespread applications such as for preservation of cosmetic products, anti-acne products, deodorants and shampoos. Further such polypeptides may be use in contact lens products.

### **Food and Feed**

Further conjugated enzymes or polypeptides with reduced allergenicity according to the invention may advantageously be used in the manufacturing of food and feed.

### Proteases

The gluten in wheat flour is the essential ingredient responsible for the ability of flour to be used in baked foodstuffs. Proteolytic enzymes are sometimes needed to modify the gluten phase of the dough, e.g. a hard wheat flour can be softened with a protease.

Neutrase® is a commercially available neutral metallo protease that can be used to ensure a uniform dough quality and bread texture, and to improve flavour. The gluten proteins is degraded either moderately or more extensively to peptides, whereby close control is necessary in order to avoid excessive softening of the dough.

Proteases are also used for modifying milk protein.

To coagulate casein in milk when producing cheese proteases such as rennet or chymosin may be used.

In the brewery industry proteases are used for brewing with unmalted cereals and for controlling the nitrogen content.

In animal feed products proteases are used so to speak to expand the animals digestion system.

### Lipases

The application of lipase in the baking industry is rather new. Addition of lipase results in improved dough properties and an improved breadmaking quality in terms of larger volume, improved crumb structure and whiter crumb colour. The observed effect can be explained by a mechanism where the lipase changes the interaction between gluten and some lipids fragment during dough mixing. This results in an improved gluten network.

The flavour development of blue roan cheeses (e.g. Danablu), certain Italian cheese types and other dairy products containing butter fat are dependent on the degradation of milk fat into free fatty acids. Lipases may be used for developing flavour in such products.

In the oil- and fat producing industry lipases are used e.g. to minimize the amount of undesirable side-products, to modify fats by interesterification, and to synthesis of esters.

### Oxidoreductases

Further oxidoreductases with reduced allergenicity according to the invention may advantageously be used in the manufacturing of food and feed.

Several oxidoreductases are used for baking, glucose oxidase,

lipoxygenase, peroxidase, catalase and combinations hereof. Traditionally, bakers strengthen gluten by adding ascorbic acid and potassium bromate. Some oxidoreductases can be used to replace bromate in dough systems by oxidation of free sulfhydryl units in gluten proteins. Hereby disulphide linkages are formed resulting in stronger, more elastic doughs with greater resistance.

Gluzyme<sup>TM</sup> (Novo Nordisk A/S) is a glucose oxidase preparation with catalase activity that can be used to replace bromate. The dough strengthen is measured as greater resistance to mechanical shock, better oven spring and larger loaf volume.

#### Carbohydrases

Flour has varying contents of amylases leading to differences in the baking quality. Addition of amylases can be necessary in order to standardize the flour. Amylases and pentosanases generally provide sugar for the yeast fermentation, improve the bread volume, retard retrogradation, and decrease the staling rate and stickiness that results from pentosan gums. Examples of carbohydrases are given below.

Certain maltogenic amylases can be used for prolonging the shelf-life of bread for two or more days without causing gumminess in the product. Selectively modifies the gelatinized starch by cleaving from the non-reducing end of the starch molecules, low molecular weight sugars and dextrans. The starch is modified in such a way that retrogradation is less likely to occur. The produced low-molecular-weight sugars improve the baked goods' water retention capacity without creating the intermediate-length dextrans that result in gumminess in the finished product. The enzyme is inactivated during bread baking, so it can be considered a processing aid which does not have to be declared on the label. Overdosing of Novamyl can almost be excluded.

The bread volume can be improved by fungal  $\alpha$ -amylases which



further provide good and uniform structure of the bread crumb. Said  $\alpha$ -amylases are endoenzymes that produce maltose, dextrins and glucose. Cereal and some bacterial  $\alpha$ -amylases are inactivated at temperatures above the gelatinization temperature of starch, therefore when added to a wheat dough it results in a low bread volume and a sticky bread interior. Fungamyl has the advantage of being thermolabile and is inactivated just below the gelatinization temperature.

Enzyme preparations containing a number of pentosanase and hemi-cellulase activities can improve the handling and stability of the dough, the freshness, the crumb structure and the volume of the bread.

By hydrolyzing the pentosans' fraction in flour, it will lose a great deal of its water-binding capacity, and the water will then be available for starch and gluten. The gluten becomes more pliable and extensible, and the starch gelatinize more easily. Pentosanases can be used in combination with or as an alternative to emulsifiers.

Further carbohydrases are utilized for producing syrups from starch, which are widely used in soft drinks, sweets, meat products, dairy products, bread products, ice cream, baby food, jam etc.

The conversion of starch is normally carried out three steps. First the starch is liquefied, by the use of  $\alpha$ -amylases. Maltodextrins, primarily consisting of oligosaccharides and dextrins, are obtained.

The mixture is then treated with an amyloglucosidase for hydrolyzing the oligosaccharides and dextrins into glucose. In this way a sweeter product is obtained. If high maltose syrups are desired,  $\beta$ -amylases alone or in combination with a pullulanase (de-branching enzyme) may be used.

The glucose mixture can be made even sweeter by isomerization to fructose. For this an immobilized glucose isomerase can be used.

In the sugar industry, it is common practice to speed up the break-down of present starch in cane juices. Thereby the starch content in the raw sugar is reduced, and filtration at the refinery facilitated.

Furthermore dextranases are used to break down dextran in raw sugar juices and syrups.

In the alcohol industry  $\alpha$ -amylases are advantageously used for thinning of starch in distilling mashes.

In the brewing industry  $\alpha$ -amylases are used for adjunct liquefaction.

In the dairy industry  $\beta$ -galactosidases (lactase) are used when producing low lactose milk for persons suffering from lactose malabsorption.

When flavoured milk drinks are produced from lactase-treated milk, the addition of sugar can be reduced without reducing the sweetness of the product.

In the production of condensed milk, lactose crystallization can be avoided by lactase treatment, and the risk of thickening caused by casein coagulation in lactose crystals is thus reduced.

When producing ice cream made from lactase-treated milk (or whey) no lactose crystals will be formed and the defect, sandiness, will not occur.

Further, xylanases are known to be used within a number of food/feed industrial applications as described in WO 94/21785 (Novo Nordisk A/S).

$\alpha$ -amylases are used in the animal feed industry to be added to cereal-containing feed to improve the digestibility of starch.

#### Anti-microbial polypeptides

Certain bacteriolytic enzymes may be used e.g. to wash carcasses in the meat packing industry (see US patent no. 5,354,681 from Novo Industri A/S)

#### Transferases

Transglutaminases with reduced allergenicity according to the invention may advantageously be used in the manufacturing of food and feed.

Transglutaminases has the ability to crosslinking protein.

This property can be used for gelling of aqueous phases containing proteins. This may be used for when producing of spreads (DK patent application no. 1071/84 from Novo Nordisk A/S).

Transglutaminases are used for improvement of baking quality of flour e.g. by modifying wheat flour to be used in the preparation of cakes with improved properties, such as improved taste, dent, mouth-feel and a higher volume (see JP 1-110147).

Further producing paste type food material e.g. used as fat substitution in foods as ice cream, toppings, frozen desserts, mayonnaises and low fat spreads (see WO 93/22930 from Novo Nordisk A/S).

Furthermore for preparation of gels for yoghurt, mousses, cheese, puddings, orange juice, from milk and milk-like products, and binding of chopped meat product, improvement of taste and

texture of food proteins (see WO 94/21120 and WO 94/21129 from Novo Nordisk A/S).

### Phytases

Phytases of the invention may advantageously be used in the manufacturing of food, such as breakfast cereal, cake, sweets, drink, bread or soup etc., and animal feed.

Phytases may be used either for exploiting the phosphorus bound in the phytate/phytic acid present in vegetable protein sources or for exploiting the nutritionally important minerals bound in phytic acid complexes.

Microbial phytase may be added to feedstuff of monogastric animals in order to avoid supplementing the feed with inorganic phosphorus (see US patent no. 3,297,548)

Further phytases may be used in soy processing. Soyabean meal may contain high levels of the anti-nutritional factor phytate which renders this protein source unsuitable for application in baby food and feed for fish, calves and other non-ruminants, since the phytate chelates essential minerals present therein (see EP 0 420 358).

Also for baking purposes phytases may be used. Bread with better quality can be prepared by baking divided pieces of a dough containing wheat flour etc. and phytase (see JP-0-3076529-A)

A high phytase activity koji mold is known to be used for producing refined sake (see JP-0-6070749-A).

## **Textile applications**

### Proteases

Proteases are used for degumming and sand-washing of silk.

### Lipases

Lipases are used for removing fatty matter containing hydrophobic esters (e.g. triglycerides) during the finishing of textiles (see e.g. WO 93/13256 from Novo Nordisk A/S).

### Oxidoreductases

In bleach clean-up of textiles catalases may serve to remove excess hydrogen peroxide.

### Carbohydrases

Cellulolytic enzymes are widely used in the finishing of denim garments in order to provide a localized variation in the colour density of the fabric (Enzyme facilitated "stone wash").

Also cellulolytic enzymes find use in the bio-polishing process. Bio-Polishing is a specific treatment of the yarn surface which improves fabric quality with respect to handle and appearance without loss of fabric wettability. Bio-polishing may be obtained by applying the method described e.g. in WO 93/20278.

During the weaving of textiles, the threads are exposed to considerable mechanical strain. In order to prevent breaking, they are usually reinforced by coating (sizing) with a gelatinous substance (size). The most common sizing agent is starch in native or modified form. A uniform and durable finishing can thus be obtained only after removal of the size from the fabric, the so called desizing. Desizing of fabrics sized with a size containing starch or modified starch is preferably facilitated by use of amylolytic enzymes.

**Oral and dermal pharmaceuticals****Proteases**

Different combinations of highly purified proteases (e.g. Trypsin and Chymotrypsin) are used in pharmaceuticals to be taken orally, and dermal pharmaceuticals for combating e.g. inflammations, edemata and injuries.

**Leather production****Transferase**

Transglutaminase is known to be used for casein finishing of leather by acting as a hardening agent (see WO 94/13839 from Novo Nordisk).

**Hard surface cleaning**

Cleaning of hard surfaces e.g. in the food industry is often difficult, as equipment used for producing dairies, meat, sea food products, beverages etc. often have a complicated shape. The use of surfactant compositions in the form gels and foams comprising enzymes have proven to facilitate and improve hard surface cleaning. Enzymes, which advantageously may be added in such surfactant compositions, are in particular proteases, lipases, amylases and cellulases.

Such hard surface cleaning compositions comprising enzymes may also advantageously be used in the transportation sector, for instance for washing cars and for general vessel wash.

Finally the invention relates to the use of the conjugate of the invention or a composition of the invention in products comprising polypeptides.

First of all the conjugate or compositions of the invention can advantageously be used for personal care products, such as hair



care and hair treatment products. This includes products such as shampoo, balsam, hair conditioners, hair waving compositions, hair dyeing compositions, hair tonic, hair liquid, hair cream, shampoo, hair rinse, hair spray.

Further oral care products are contemplated such as dentifrice, mouth washes, chewing gum.

Also skin care products and cosmetics are contemplated, such as skin cream, skin milk, cleansing cream, cleansing lotion, cleansing milk, cold cream, cream soap, nourishing essence, skin lotion, milky lotion, calamine lotion, hand cream, powder soap, transparent soap, sun oil, sun screen, shaving foam, shaving cream, baby oil lipstick, lip cream, creamy foundation, face powder, powder eye-shadow, powder, foundation, make-up base, essence powder, whitening powder.

Also for contact lenses hygiene products the conjugate of the invention can be used advantageously. Such products include contact lenses cleaning and disinfection products.

The use for detergents such as washing powder, soap, soap bars, liquid soap are also contemplated.

## **METHODS AND MATERIALS**

### Materials:

Methoxypolyethylene glycol 15.000 (mPEG from Shearwater)

Methoxypolyethylene glycol 5.000 (mPEG from Fluka)

Subtilisin Novo (from Novo Nordisk A/S)

Carezyme© core (from Novo Nordisk A/S)

Succinyl-Alanine-Alanine-Proline-Phenylalanine-para-nitroanilide (Suc-AAPF-pNP) Sigma no. S-7388, Mw 624.6 g/mole.

### ELISA reagents:

Horse Radish Peroxidase labeled anti-rat-Ig ( Dako, DK, P162, #

031; dilution 1:1000).

Biotin-labelled mouse-anti-rat-IgE (Zymed 03-9740; dilution 1:1000).

Streptavidin-horse radish peroxidase (Kirkegård & Perry 14-30-00; dilution 1:1000).

#### Solutions:

Stop-solution (DMG-buffer)

Sodium Borate, borax (Sigma)

3,3-Dimethyl glutaric acid (Sigma)

CaCl<sub>2</sub> (Sigma)

Tresyl chloride (2,2,2-triflouroethansulfonyl chloride) (Fluka)

Tween 20: Poly oxyethylene sorbitan mono laurate (Merck cat no. 822184)

1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Fluka)

N-Hydroxy succinimide (Fluka art. 56480)

Phosgene (Fluka art. 79380)

Lactose (Merck 7656)

PMSF (phenyl methyl sulfonyl flouride) from Sigma

#### Colouring substrate:

OPD: o-phenylene-diamine, (Kementec cat no. 4260)

#### Test Animals:

Brown Norway rats (from Charles River, DE)

#### Equipment:

XCEL II (Novex)

ELISA reader (UVmax, Molecular Devices)

HPLC (Waters)

PFLC (Pharmacia)

Superdex-75 column, Mono-Q, Mono S from Pharmacia, SW.

SLT: Fotometer from SLT LabInstruments

Size-exclusion chromatograph (Spherogel TSK-G2000 SWG).

Size-exclusion chromatograph (Superdex 200, Pharmacia, SW)

Amicon Cell

### Protease activity

#### Analysis with Suc-Ala-Ala-Pro-Phe-pNA:

Proteases cleave the bond between the peptide and p-nitroaniline to give a visible yellow colour absorbing at 405 nm.

Buffer: e.g. Britton and Robinson buffer pH 8.3

Substrate: 100 mg suc-AAPF-pNA is dissolved into 1 ml dimethyl sulfoxide (DMSO). 100 µl of this is diluted into 10 ml with Britton and Robinson buffer.

#### Analysis

The substrate and protease solution is mixed and the absorbance is monitored at 405 nm as a function of time and  $ABS_{405 \text{ nm/min}}$ . The temperature should be controlled (20-50°C depending on protease). This is a measure of the protease activity in the sample.

#### Analysis with Casein

The analysis is performed according to standard Novo Nordisk procedures described in AF 220 which are hereby included as reference (and available of request).

#### Carezyme® activity

Enzymatic activity was measured as release of blue dye from azurine-crosslinked HE-cellulose (Cellazyme-C®). The reaction was carried out at 40°C in 20 mM Na-phosphate pH 7 for 10 minutes. Release of dye was monitored by reading the absorbance at 595 nm in a UVmax® Elisa-reader. In addition, cellulytic activity was measured as described in "EAL-SM-0373.01/01" (available from Novo Nordisk on request).

ELISA Ig/IgE test system

A three layer sandwich ELISA is used to determine relative concentrations of total Ig or IgE antibodies.

The immunizing molecule is used as coating antigen with 10 µg per ml and 50 µl per well, in neutral phosphate buffer, incubated overnight at 4°C. All remaining binding spots on the well surface are blocked in 2 % skim milk, 200 µl per well in phosphate buffer for at least 30 minutes at room temperature (RT). All sera to be tested with this antigen are added at 50 µl per well to this plate using a 8-channel pipette in dilution series from 10 x diluted followed by 3-fold dilutions. Dilutions are made in phosphate buffer with 0.5% skim milk and 0.05% Tween20, incubated 2 hours on agitation platform at RT. The "tracer" molecule(s) is(are) streptavidin-horse-radish peroxidase (when testing for total Ig) and biotin labelled mouse-anti-rat IgE coupled with Streptavidin horse-radish peroxidase (when testing for IgE). 50 µl tracer molecules(s) per well diluted 2000 times in phosphate buffer with 0.5 % skim milk and 0.05% Tween20 are incubated for 2 hours on an agitation platform at room temperature (RT). Controls (blanks) are identical sequence but without rat sera. 50 µl per well Streptavidin horse radish peroxidase, diluted 2000 times is incubated 1 hour on an agitation platform.

The colouring substrate (50 µl per well) is OPD (6 mg) and H<sub>2</sub>O<sub>2</sub> (4 µl of a 30% solution) per 10 ml citrate buffer pH 5.2. The reaction is stopped using 100 µl per well 2 N H<sub>2</sub>SO<sub>4</sub>. All readings on SLT are performed at 486 nm and 620 nm as reference. Data is calculated and presented in Lotus.

Intratracheal (IT) stimulation of rats

For IT administration of molecules disposable syringes with a 2½" long metal probe are used. This probe is instilled in the trachea of the rats approximately 1 cm below the epiglottis,

and 0.1 ml of a solution of the molecules is deposited. The animals are stimulated 4 times, with 5 days between the last stimulation and exsanguination.

The test animals are Brown Norway rats (BN) in groups of 10. Weight at time of start is more than 250 grams and at termination approximately 450 grams.

#### Determination of the molecular weight

Electrophoretic separation of proteins was performed by standard methods using 4-20% gradient SDS poly acrylamide gels (Novex). Proteins were detected by silver staining. The molecular weight was measured relative to the mobility of Mark-12® wide range molecular weight standards from Novex.

### **EXAMPLES**

#### **EXAMPLE 1**

##### Preparation of mPEG 15,000 chloroformiate

10 gram mPEG 15,000 was suspended in 60 ml toluene of which 15 ml was distilled off to remove any trace of water. After cooling to ambient temperature some precipitate formed, which re-dissolved upon addition of 10 ml anhydrous dichloromethane. 1.7 ml phosgene (1.93 M in toluene 5 eqv.) was added without any detectable reaction. After 14 hours at ambient temperature the mixture was evaporated to dryness to remove phosgene. To improve the crystalline structure it is preferable to re-crystallise from toluene (dry 5 ml/ g mPEG). Yield after filtration and drying generally exceeds 98% and activation degree is better than 90% by NMR. <sup>1</sup>H-NMR for mPEG 15,000 (CDCl<sub>3</sub>) δ 3.38 s (I= 2.6 CH<sub>3</sub> i OMe), 3.40\* dd (I = 4.5 0/00, <sup>13</sup>C satellite), 3.64 bs (I = 1364 main peak), 3.89\* dd (I = 4.8 0/00,, <sup>13</sup>C satellite), 4.46 q\* (I = 1.8, CH<sub>2</sub> α to chloroformiat). When stored in an desiccator at 22°C a decrease

in activation degree of 40% was detected after 3 months and a new peak was detected at  $\delta$  4.37. When stored for 5 months a decrease of 70% was detected and the same peak at  $\delta$  4.37 was seen. When stored at  $-18^{\circ}\text{C}$  no change was detected after 3 months.

## EXAMPLE 2

### Preparation of mPEG 5,000 chloroformiate

10 gram mPEG 5,000 was suspended in 60 ml toluene of which 15 ml was distilled off to remove any trace of water. After cooling to ambient temperature some precipitate formed, which redissolved upon addition of 10 ml anhydrous dichloromethane. 15.2 ml phosgene (1.93 M in toluene 5 eqv.) was added without any detectable reaction. After 14 hours at ambient temperature the mixture was evaporated to dryness to remove phosgene. To improve the crystalline structure the mixture was re-crystallised.

## EXAMPLE 3

### Conjugation of protease with mPEG 15,000-chloroformiate

To a solution (10 ml) of 100 mg of highly purified Subtilisin Novo in 0.1 M Borate (pH 9.5, 0.5 M NaCl) 3 x 550 mg of methoxypolyethylene glycol-15,000-chloroformiate was added sequential at 0, 30 & 75 minutes.

The resulting Subtilisin Novo-mPEG-15,000 conjugate was purified by size-exclusion chromatography using a Superdex-75 column in an HPLC-system.

The residual activity of the conjugate was assessed by using suc-AAPF-pNP and casein as substrates:

■ peptide substrate : 95%



■ CM-casein : 60%

#### EXAMPLE 4

##### Conjugation of a protease with mPEG 5,000 chloroformiate

To a solution (10 ml) of 100 mg of highly purified Subtilisin Novo in 0.1 M Borate (pH 9.5) and 50% dimethylformamide (DMF) 3 x 367 mg of methoxypolyethylene glycol 5,000 chloroformiate was added sequential at 0, 30 & 75 minutes.

The reaction was performed at ambient temperature using magnetic stirring. At time 120 minutes the reaction was terminated by addition of 0.5 ml of 2 M Glycine.

The resulting Subtilisin Novo-mPEG-5,000 conjugate was purified by size-exclusion chromatography using a Superdex-75 column in an HPLC-system.

The residual activity of the conjugate is assessed by using suc-AAPF-pNP as substrate. The conjugated retained activity towards the peptide substrate.

#### EXAMPLE 5

##### Conjugation of a cellulase with mPEG 5,000 chloroformiate

The catalytic core-domain of Carezyme© was prepared according to Boisset, C. et al. (1995), FEBS Lett. 376, p. 49-52.

To a solution (12 ml) of 100 mg of the purified Carezyme© core in 0.1 M NaHCO<sub>3</sub>, 0.2 M Na-Borate (pH 8.5) 1.5 g of methoxypolyethylene glycol 5,000 chloroformiate was added.

The reaction was performed at ambient temperature using magnetic stirring. At time 30 minutes the reaction was terminated by addition of 1 ml of 2 M Glycine.

The resulting Cellulase-core-domain-mPEG-5,000 conjugate was purified by desalting in 0.05 M NaHCO<sub>3</sub> and concentrated to the original volume using an Amicon Cell.

The obtained conjugate was PEGylated a second time using 1.0 g of methoxypolyethylene glycol 5,000 chloroformate at otherwise identical conditions.

The final two times PEGylated Carezyme© core-mPEG-5,000 conjugate was purified by desalting in water and concentrated to approximately 1 mg/ml of protein using an Amicon Cell. The conjugate was stored at 4°C.

#### EXAMPLE 6

##### IT-Studies in Brown Norway Rats.

To assess the reduction in allergenicity of enzymes resulting from modification according to the invention Carezyme© core (unmodified), Carezyme© core-PEG 5,000 (modified, Example 5), Subtilisin Novo (unmodified), and Subtilisin Novo-PEG 15,000 (modified, Example 3), Glycine-PEG 15,000, 0.9% NaCl (control) was introduced intratracheally (IT) into Brown Norway rats (BN).

Sera from immunized BN rats were tested in the ELISA assay (described above) to elucidate whether the molecules had penetrated the lung epithelias and activated the immune response system giving rise to a total Ig (assessed as IgG) and IgE response.

The results of the IT tests are shown in Figure 1 to 5.

As can be seen from the Figures the amount of total Ig (assessed as IgG) and IgE (allergic response) of BN rats exposed intratracheally with the modified polypeptides (i.e. PEGylated Carezyme© core and PEGylated Subtilisin Novo) has

been reduced in comparison to the BN rats having been exposed intratracheally with the parent unmodified polypeptides.

As will be apparent to those skilled in the art, in the light of the foregoing disclosure, many alterations and modifications are possible in the practice of this invention without departing from the spirit or scope thereof. Accordingly, the scope of the invention is to be construed in accordance with the substance defined by the following claims.

As will be apparent to those skilled in the art, in the light of the foregoing disclosure, many alterations and modifications are possible in the practice of this invention without departing from the spirit or scope thereof. Accordingly, the scope of the invention is to be construed in accordance with the substance defined by the following claims.

**PATENT CLAIMS**

1. A process of producing polyalkylene oxide-polypeptide conjugates with reduced allergenicity using a polyalkylene oxide (PAO) as the starting material, comprising the steps of

- a) generating a polyalkylene oxide halogen formiate, and
- b) conjugating at least one polyalkylene oxide halogen formiate to attachment groups on the polypeptide.

2. The process according to claim 1, wherein the polyalkylene oxide (PAO) is an polyalkylene glycol (PAG) or and methoxypolyalkylene glycol (mPAG).

3. The process according to claim 2, wherein the PAG or mPAG is an polyethylene glycol (PEG) or methoxypolyethylene glycol (mPEG).

4. The process according to any of claims 1 to 3, wherein the polypeptide is a protein.

5. The process according to any of the claims 1 to 4, wherein the polypeptide has anti-microbial activity.

6. The process according to claims 4 and 5, wherein the protein has biological activity.

7. The process according to any of claims 4 to 6, wherein the protein has enzymatic activity.

8. The process according to claim 7, wherein the enzyme is a protease, a lipase, a transferase, a carbohydrase, an oxidoreductase, or a phytase.

9. The process according to any of claims 1 to 8, wherein the

polyalkylene oxide halogen formiate is a polyalkylene oxide chloroformiate.

10. The process according to claim 9, wherein the polyalkylene oxide chloroformiate is a polyalkylene glycol chloroformiate.

11. The process according to claim 10, wherein the polyalkylene glycol chloroformiate is a polyethylene glycol chloroformiate.

12. The process according to claim 10, wherein the polyethylene glycol chloroformiate is a methoxypolyethylene glycol chloroformiate.

13. The process according to any of claims 1 to 12, wherein step a) is mediated by phosgene ( $\text{Cl}_2\text{-C=O}$ ).

14. The process according to any of claims 1 to 13, wherein the attachment groups are at least one selected from the groups including amines, alcohols, phenols, or carboxylic acids.

15. The process according to claim 14, wherein the polyalkylene oxide halogen formiate attach to the side chain serine, threonine, tyrosine, lysine, arginine, aspartate, glutamate in the polypeptide chain.

16. An activated polyalkylene oxide capable of linking to attachment groups on a polypeptide, whose activated PAO has the generic structure



wherein

R1 is hydrogen, methyl, hydroxyl or methoxy,

R2 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

R3 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

R4 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,  
a is an integer between 1 and 1000,  
b is an integer between 0 and 1000,  
c is an integer between 0 and 1000, and  
Y is a halogen or nitrile

17. The activated PAO according to claim 16, wherein the halogen is Cl, Br, or F.

18. The activated PAO according to claims 16 and 17, wherein R2, R3 and R4 is CH<sub>2</sub>-CH<sub>2</sub>, CH<sub>2</sub>-CH-CH<sub>3</sub>, or CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>.

19. The activated PAO according to any of claims 16 to 18, wherein the molecular weight lies between 1 kDa and 60 kDa, such as between 2 kDa and 35 kDa, especially between 2 kDa and 25 kDa.

20. A polyalkylene oxide-polypeptide conjugate having the general formula

$(R1 - (O-R2)_a - (O-R3)_b - (O-R4)_c - O - C=O - X -)_n$  polypeptide

wherein

R1, R2, R3, R4, a, b and c, are defined in claim 16, n is an integer between 1 and 100, and X is a coupling group between the polymer and an polypeptide.

21. The conjugate according to claim 20 being produced by the process according to claims 1 to 15.

22. The conjugate according to claims 20 and 21, wherein X is an attachment group selected from the group including amines, alcohols, phenols, and/or carboxylic acid group on the polypeptide.

23. The conjugate according to any of claims 20 to 22, wherein



R2, R3 and R4 are  $\text{-CH}_2$ ,  $\text{CH}_2\text{-CH-CH}_3$ , or  $\text{CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_2$ .

24. The conjugate according to any of claims 20 to 23, wherein the polypeptide is a protein.

25. The conjugate according to any of claims 20 to 24, wherein the polypeptide has anti-microbial activity.

26. The conjugate according to claims 24 and 25, wherein the protein has biological activity.

27. The conjugate according to any of claims 24 to 26, wherein the protein has enzymatic activity.

28. The conjugate according to claim 27, wherein the enzyme is a protease, a lipase, a transferase, a carbohydrase, an oxidoreductase, or a phytase.

29. The conjugate according to any of claims 20 to 28, wherein the total molecular weight lies 50 kDa and 250 kDa, preferably between 80 kDa and 200 kDa.

30. The conjugate according to any of claims 20 to 29, wherein the molecular weight of the PAO lies between 1 kDa and 60 kDa, such as between 2 kDa and 35 kDa, especially between 2 kDa and 25 kDa.

31. A composition comprising a polyalkylene oxide-polypeptide conjugate according to any of claims 20 to 30, which further comprises other enzymes/polypeptides and/or ingredients normally used in detergents, including soap bars, household articles, agrochemicals, personal care products, including cleaning preparations for contact lenses and skin and hair cleaning preparations, cosmetics, toiletries, oral and dermal pharmaceuticals, composition for treating textiles, compositions for cleaning hard surfaces, and compositions used for manufacturing food and feed.

32. The composition according to claim 31, wherein one, more or all of said other enzymes/polypeptides are modified according to any of claims 1 to 15 or are conjugates according to any of claims 16 to 30.

33. The composition according to claims 31, comprising at least one enzyme/polypeptide from the group comprising proteases, lipases, oxidoreductases, carbohydrases, transferases, such as transglutaminases, anti-microbial polypeptides, and phytases.

34. Use of a conjugate according to any of claims 20 to 30 or compositions according to claims 31 and 33, in industrial products comprising a polypeptide.

35. The use according to claim 34 in personal care products.

36. The use according to claim 35 for hair care or hair treatment products.

37. The use according to claim 36 for shampoo, balsam, hair conditioners, hair waving compositions, hair dyeing compositions, hair tonic, hair liquid, hair cream, shampoo, hair rinse, hair spray.

38. The use according to claim 35 in oral care products.

39. The use according to claim 38 for dentifrice, mouth washes, chewing gum.

40. The use according to claim 35, in skin care products.

41. The use according to claim 40, in skin cream, skin milk, cleansing cream, cleansing lotion, cleansing milk, cold cream, cream soap, nourishing essence, skin lotion, milky lotion, calamine lotion, hand cream, powder soap, transparent soap, sun

oil, sun screen, shaving foam, shaving cream, and baby oil.

42. The use according to claim 35 in cosmetics.

43. The use according to claim 42 for lipstick, lip cream, creamy foundation, face powder, powder eye-shadow, powder, foundation, make-up base, essence powder, whitening powder.

44. The use according to claim 34 for contact lenses hygiene products.

45. The use according to claim 44 for contact lenses cleaning and disinfection products.

46. The use according to claim 34 in detergents.

47. The use according to claim 46 in washing powder.

48. The use according to claim 46 in liquid detergents.

49. The use according to claim 46 in dishwash detergents.

50. The use according to claim 46 for soap, soap bars, liquid soap.

51. The use according to claim 34, in oral and dermal pharmaceuticals.

52. The use according to claim 34, in agrochemicals.

53. The use according to claim 34, in food or feed.

54. The use according to claim 53, in baking products.

55. The use according to claim 34, in products for processing textiles.

56. The use according to claim 34, in compositions for cleaning hard surfaces.

57. A method for reducing the allergenicity of industrial polypeptides by using the process according to claims 1 to 15.

IT-study in BN rats.  
serum IgG and IgE antibody to Carezyme Core (CC)/ChiPEG5000.

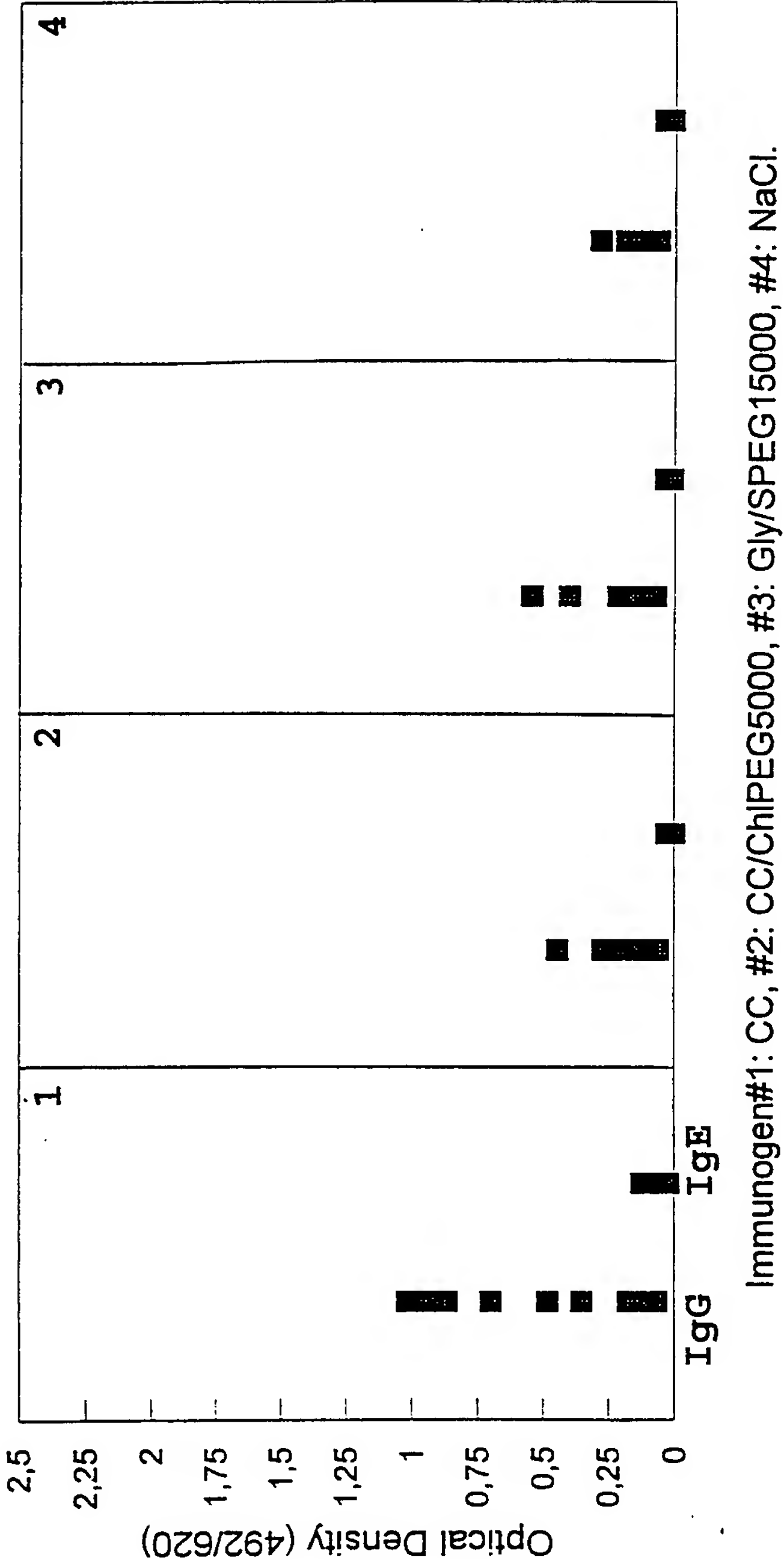


Fig. 1

IT-study in BN rats.  
serum IgG and IgE antibody to Carezyme Core (CC).

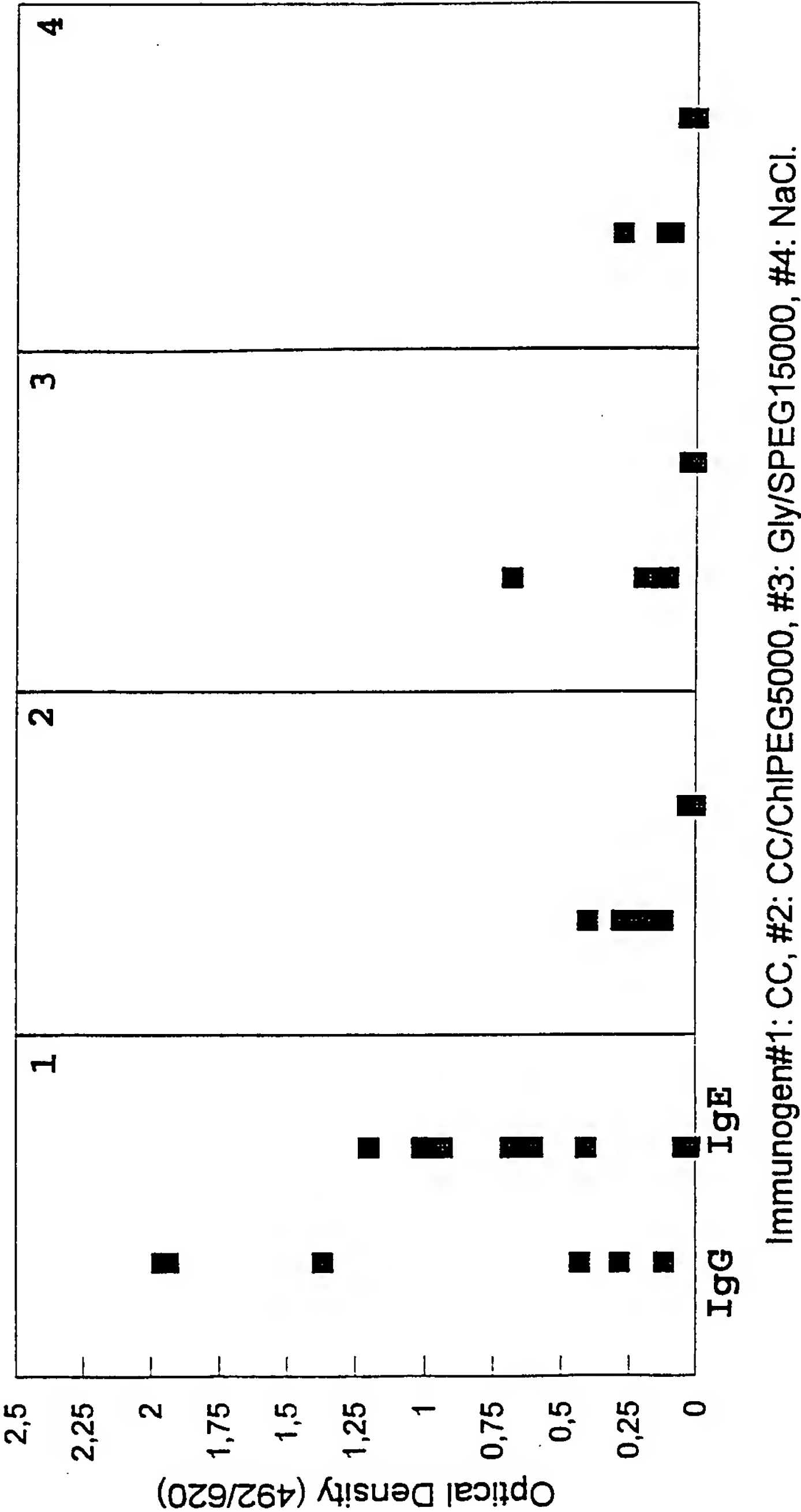


Fig. 2



IT-study in BN rats..  
serum IgG and IgE antibody to Sub.Novo (SN).

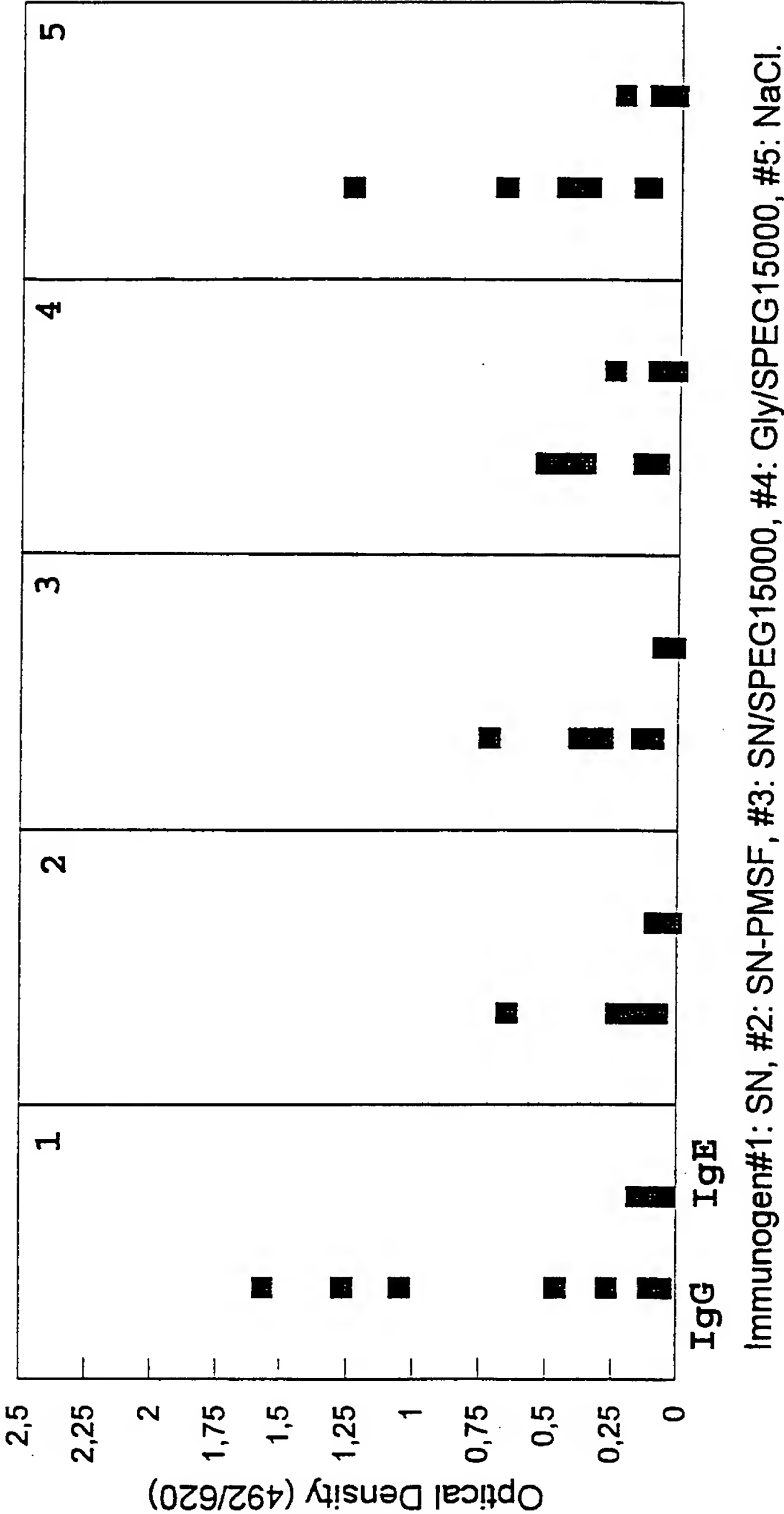


Fig. 3

IT-study in BN rats.  
serum IgG and IgE antibody to Sub.Novo (SN) (+PMSF).

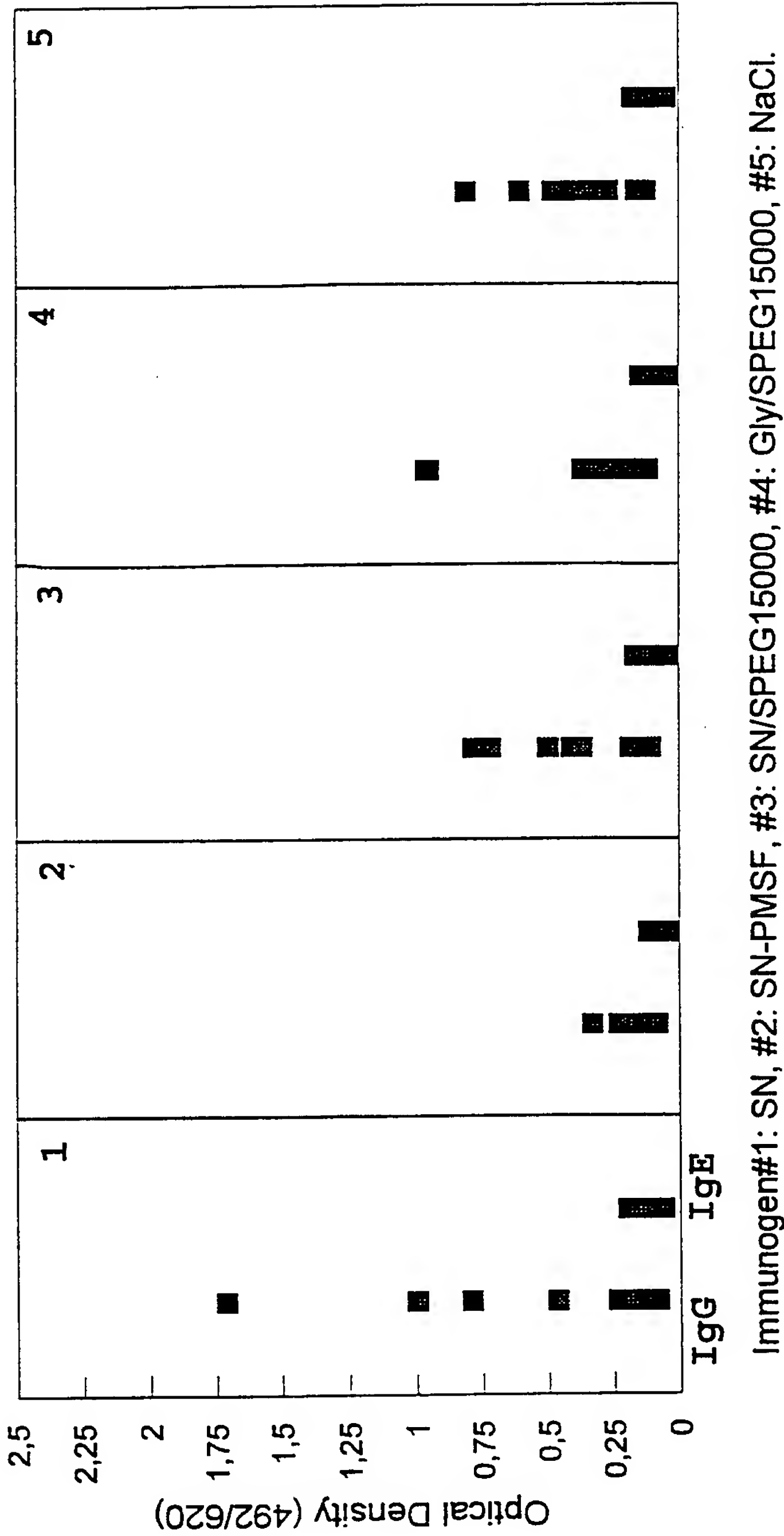
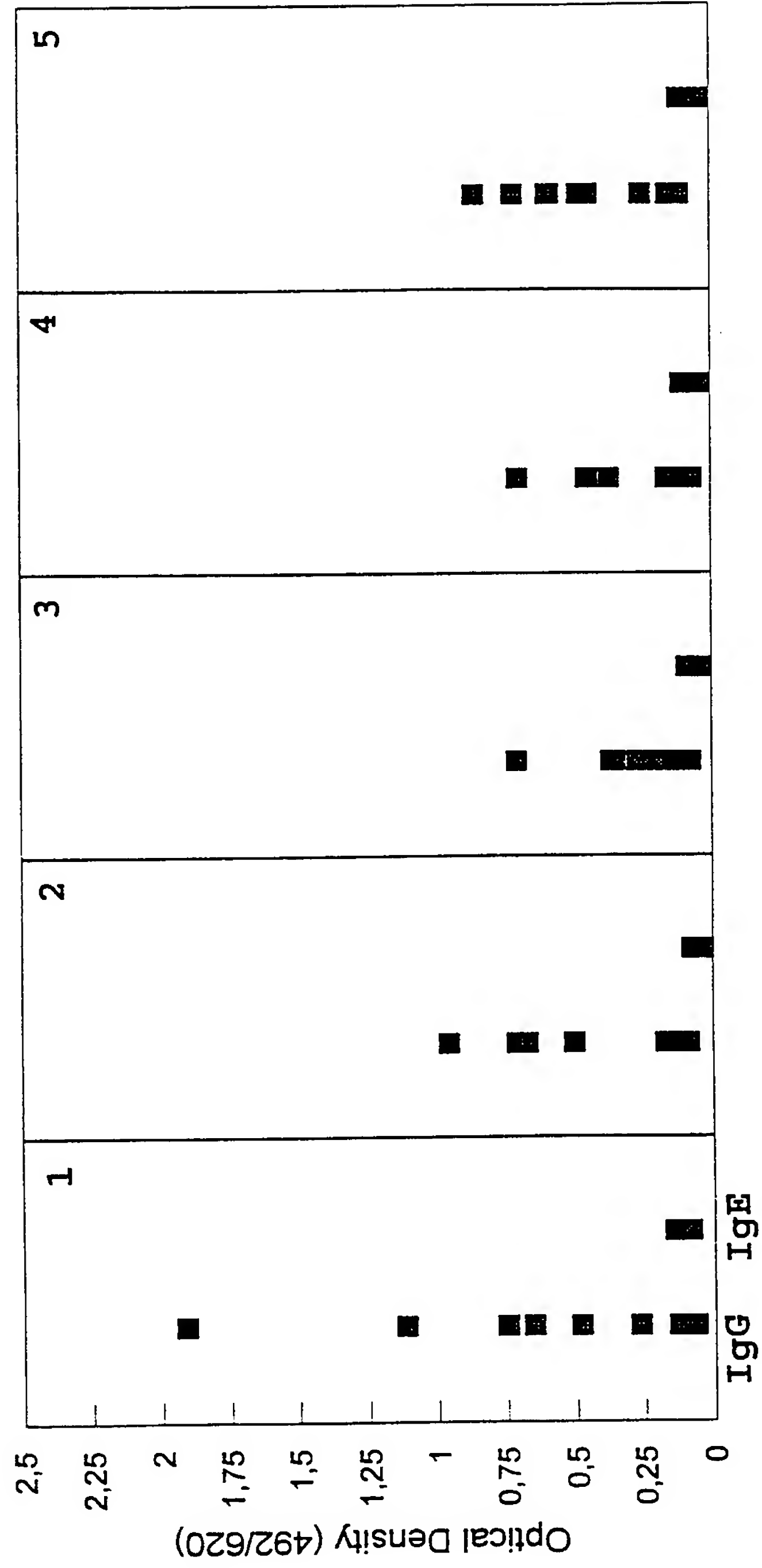


Fig. 4

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# IT-study in BN rats.

serum IgG and IgE antibody to Sub.Novo (SN)/SPEG15000.



Immunogen#1: SN, #2: SN-PMSF, #3: SN/SPEG15000, #4: Gly/SPEG15000, #5: NaCl.

Fig. 5

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 96/00249

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 17/08

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, EMBASE, CA, CLAIMS, WPI, JAPIO

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9607670 A1 (SURFACE ENGINEERING TECHNOLOGIES DIVISION OF INNERDYNE, INC.), 14 March 1996 (14.03.96), see page 3 and claims --	1-57
X	EP 0557199 A1 (TRANSGENE S.A.), 25 August 1993 (25.08.93), page 2, line 38 - line 41, claims --	1-57
X	DD 287951 A5 (AKADEMIE DER WISSENSCHAFTEN DER DDR), 14 March 1991 (14.03.91), see claims --	1-57
X	EP 0632082 A1 (HEYLECINA, SOCIETE ANONYME), 4 January 1995 (04.01.95), see page 11 and claims --	1-57

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

26 August 1996

Date of mailing of the international search report

02 -09- 1996

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 96/00249

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 9101758 A1 (DEBIOPHARM S.A.), 21 February 1991 (21.02.91), see example 1 and claims --	1-57
A	WO 9404193 A1 (ENZON, INC.), 3 March 1994 (03.03.94), abstract, see example 2 and claims --	1-57
A	WO 9417039 A1 (ENZON, INC.), 4 August 1994 (04.08.94), see example 2 and page 1 --	1-57
A	DE 3440141 A1 (ECKERT, HEINER), 7 May 1986 (07.05.86), see page 6 and claims 1,7 --	1-57
A	US 4179337 A (F.F. DAVIS ET AL), 18 December 1979 (18.12.79), see whole document -- -----	1-57

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31/07/96

International application No.

PCT/DK 96/00249

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